Research Article

For reprint orders, please contact: reprints@futuremedicine.com

Regenerative Medicine

Astragalus and human mesenchymal stem cells promote wound healing by mediating immunomodulatory effects through paracrine signaling

Jiaqi Wang[‡,1], Dandan Zhang[‡,2], Ying Zhu[‡,3], Xiumei Mo[4], Patrick C McHugh[*,5] & Qiang Tong[**,6]

[1]Clinical Research Center, Changhai Hospital, Shanghai, 200433, China

[2]Arachna Skin Biotechnology Center, Eston Cell Technology (Shanghai) Co. Ltd, Shanghai, 201611, China

[3]Department of Respiratory & Critical Care Medicine, Seventh Medical Center of Chinese PLA General Hospital, Beijing, 100700, China

[4]Shanghai Engineering Research Center of Nano-Biomaterials and Regenerative Medicine, College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, Shanghai, 201620, China

[5]Centre for Biomarker Research, School of Applied Sciences, University of Huddersfield, HD1 3DH, UK

[6]Department of Rheumatology & Immunology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, 200235, China

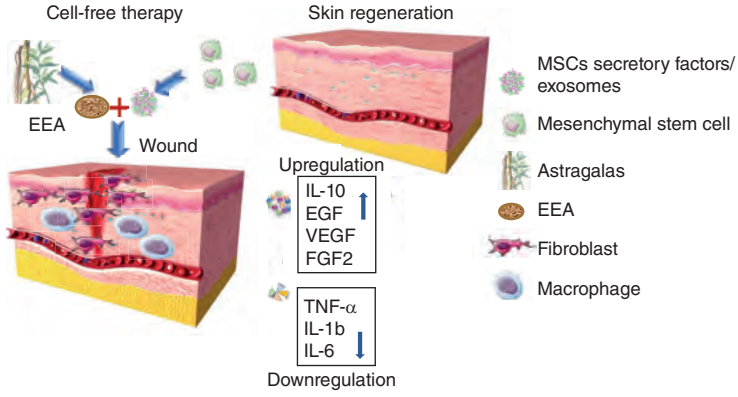
*Author for correspondence: p.c.mchugh@hud.ac.uk

**Author for correspondence: jasontong1985@outlook.com

[‡]Authors contributed equally to this paper

Background: Skin regeneration from an injury without a scar is still a challenge. **Methods:** A murine model of a skin wound was treated with a combination of extract of astragalus and exosomes of mesenchymal stem cells (MSCs). CD11b+ and CD45 macrophages were detected and levels of cytokines were tested. **Results:** The expression of growth factors VEGF, FGF2 and EGF was elevated after treatment administered to MSCs. The administration of ethanolic extract of astragalus decreased the expression of TNF-α, IL-1β and IL-6 and simultaneously increased the levels of IL-10. The combination sped up the process of wound healing. A sustained-release gel with both ingredients was developed to enhance restoration from granulation. **Conclusion**: The extract of astragalus promotes the efficacy of MSC-derived exosomes in skin repair.

Graphical abstract:



Plain language summary: Recovery from and regeneration of skin wounds are essential to maintaining epidermal function. Improving restoration and reducing scar tissue effectively need to be explored. Here, the authors investigated the potential role of extracts from the combination of an herbal plant (astragalus) and mesenchymal stem cells in wound healing. The administration of ethanolic extract of astragalus decreased the expression of inflammatory factors, increased the anti-inflammatory factor IL-10

Future Medicine

and inhibited the proliferation of fibroblasts. The authors found that the combination treatment reduced the recovery time, with a lighter scar. Finally, the authors developed a slow-release gel with the mixture to prolong the effect and promote wound repair. Ethanolic extract of astragalus could enhance the properties of mesenchymal stem cells by effectively increasing recovery speed and improving prognosis.

First draft submitted: 9 June 2021; Accepted for publication: 3 February 2022; Published online: 7 March 2022

Keywords: ethanolic extract of astragalus • mesenchymal stem cells • restoration • scar tissue • wound healing

Introduction

Skin wounds are a common form of trauma to the body; they can be defined as the breaking and a defect of skin tissue in the face of external forces [1]. A skin wound is a complex physiological phenomenon that includes regeneration [2] and the formation of granulation tissue [3,4] and hypertrophic scar tissue [5,6]. Recovery from a skin wound is essential to maintain survival and quality of life [1,4].

In general, repairing a skin wound can be divided into three stages: acute inflammation, cell proliferation and function remodeling [6,7]. Among them, cell proliferation is considered the key point of wound repair [8]. At this stage, blood vessels in normal tissue extend toward the wound under the stimulation of cytokines and form a network of new blood vessels [9]. Meanwhile, granulation tissue, composed of fibroblasts, gradually covers the entire wound [3,10]. On this basis, granulation tissue transitions into complete skin tissue [10,11]. More cells – including fibroblasts, keratinocytes and endothelial cells; extracellular matrix; cytokines; and growth factors – are involved in the following proliferation stage. The formation of granulation tissue is characteristic of this step, which takes days to weeks. This proliferative phase is crucial to skin wound repair, which provides a suitable microenvironment for skin regeneration [12]. Also, it establishes the material base for the remodeling of vascular networks [9,13]. This phase lasts for days and weeks [9,12–14]. However, several factors can interfere with the repair process, such as chronic inflammation [15], infections [16] and metabolic disorders [17]. These problems can lead to abnormal growth of granulation tissue, thereby inhibiting wound recovery [18].

Previous studies have suggested that mesenchymal stem cells (MSCs) are rich in growth factors, such as EGF [19], FGF [20], VEGFA [21], PDGF and TGF-β1, which enhance the wound-healing process [22]. These cytokines released from MSCs might accelerate wound healing by reducing deleterious tissue inflammation, inducing angiogenesis in the wound bed and reducing scarring in the repair process. Although the cytokines of MSCs have been widely studied in the repair process of skin wounds, the authors' previous research has found that solely using these cytokines as a treatment has some limitations. They only promote the growth of blood vessels and granulation tissue in the short term. On the one hand, cytokines can promote the development of granulation tissue and lead to excess accumulation of fibroblasts, resulting in excess scar tissue [23], but they remain active for only a short period. At present, there is no proven stem cell-based therapy for antifibrosis in clinical practice.

The authors' team has been focusing on screening the different herbal medications for wound healing, and the extracts produced have shown some promise. Evidence shows that plant extracts can inhibit inflammation and regulate basal cell growth, enhancing the efficiency of wound healing and reducing scar formation [24,25]. Plants such as ginkgo biloba [26], kelp and astragalus [27] have been investigated to prove the existence of these effective chemical constituents; for example, ginkgo lactone B extracted from ginkgo biloba is effective in the inhibition of inflammatory response [26]. Astragalus, also known as huangqi in China, is one of the most popular traditional Chinese medicines worldwide. It is the dried root of *Astragalus mongholicus Bunge*. It is widely used as an immune stimulant, an antioxidant, a hepatoprotectant, a diuretic, an antidiabetic, an anticancer drug and an expectorant. There are more than 100 compounds that have been isolated and identified from this herbal plant. Ethanolic extract of astragalus (EEA), also known as a flavonoid, was one of the ingredients extracted from astragalus, which was reported to have anti-inflammatory, antioxidant and immunomodulating activities [28]. Hence, the authors attempted to study the role of this component in skin wound healing.

In this study, the authors found that astragalus flavonoids, the ethanolic extract of this herbal plant, can effectively constrain local immune response and lessen the excessive proliferation of fibroblasts. The mixture of this additive and MSC-derived exosomes can significantly accelerate the process of skin restoration and reduce scar tissue formation. Moreover, using a specific gel as a carrier can provide sustained release of paracrine trophic factors of MSCs to augment efficacy.

Methods

All authors had access to the study data and reviewed and approved the final manuscript. All methods were performed in accordance with the relevant guidelines and local regulations. The study was approved by the ethics committees as required from the Changhai Hospital. Written informed consent was obtained from each patient involved or a family member.

Mice

6-week-old male nude mice and Sprague Dawley (SD) rats (bred in the Experimental Animal Center, Shanghai Second Military Medical University) were maintained in a pathogen-free animal facility for at least 1 week prior to use. The experiments were performed in accordance with the Institutional Animal Care and Use Committee guidelines of Shanghai Second Military Medical University.

Cultures of cell lines

The HEK-293T (*Homo sapiens*, Embroyo, kidney cell), 3T3 (*Mus musculus*, embroyo, fibroblast), HUVEC (*Homo sapiens*, umbilical cord, endothelial cell), Ha-cat (*Homo sapiens*, keratinocyte) and RAW264.7 (*Mus musculus*, ascites, macrophage) cell lines were purchased from the Resource Center, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences. All cell lines were maintained in DMEM (Gibco, NY, USA) supplemented with 10% fetal calf serum (FCS) and cultured at 37°C in a 5% CO_2 environment.

Cell Counting Kit 8

Human umbilical vein endothelial cells (HUVECs), Ha-cat and fibroblasts were seeded in 96-well plates and cultured for 24 h. 10 μl of the solution of CCK8 was added to each well for incubation at 37°C with 5% CO_2 for 1 h. Optical density (OD) values were then measured at a wavelength of 450 nm. The proliferative activity of HUVECs, Ha-cat and fibroblasts was detected after administration of EEA, MSCs and the combination of both agents.

MSC extraction & identification

Umbilical cord tissue was obtained from volunteers, and the blood vessels were excised along with the film and blood clots. Compared with MSCs from bone marrow and adipose tissue, Wharton's jelly (WJ)-derived MSCs possess more stemness and are easily obtained from umbilical cord tissue. The latter was collected from volunteers, and the blood vessels were excised along with the film and blood clots. WJ was separated and cut into 1 mm^3 pieces and placed in a 10 cm sterile Petri dish. This was followed by digestion with collagenase II in DMEM medium. After filtration, subculturing was carried out using DMEM containing 10% fetal bovine serum (FBS) culturing with serum-free medium from day 3. Digestion with EDTA-containing medium (final concentration 0.05 M) and washing with phosphate-buffered saline (PBS) solution were carried out on day 11 [29]. MSCs were identified by marking positive with CD73 and CD90, and negative with CD45 and HLA-DR (Supplementary Figure 1). The stirrer and ultrasonic wave generator were then switched on and the temperature was increased to 450°C and maintained for 20 min. A total of 106 hucMSCs were used and the maximum amplitude was set to 40%. The sample was placed in the device, resuspended and crushed with ultrasonication in the condition of 120 W, 15 s x 20-times with 15 s intervals. The crushed product was centrifuged at 10,500 *x* g for 15 min, and the supernatant was taken. The supernatant was purified using a YM-50 ultrafiltration tube (Millipore) and concentrated [30].

Ethanolic extract of astragalus

Accurately weighed, dried astragalus (50 g) was added to 200 ml of distilled water, ground and put aside for 4 h. This was then boiled and distilled. The distillate was collected and centrifuged (4°C, 10,500 *x* g, 15 min). The aqueous phase was taken and the precipitate discarded. A 1:1 ratio of ethanol was added, followed by mixing and concentration at 10,500 x g for 15 min. The supernatant was then concentrated with a negative-pressure centrifuge (-30 mmHg, 45°C) to about 5 ml (approximately 2 h). The standard curve of the standard preparation of EEA was used to quantify the product via the absorbance method (Supplementary Figure 2).

Production of EEA-MSC gels

Oxidized dextran (Odex) and modified gelatin (Mgel) were used to fabricate a fast-forming hydrogel without the addition of a chemical cross-linking agent. The dextran–gelatin was obtained from Dr Xiu-mei Mo of State

10.2217/rme-2021-0076

Table 1. The primers used in the real-time PCR analysis.

Gene	Forward primer (5′-3′)	Reverse primer (3′-5′)
VEGFA	TATTCAGCGGACTCACCAGC	AACCAACCTCCTCAAACCGT
FGF2	GGCTGCTGGCTTCTAAGTGT	GTCCCGTTTTGGATCCGAGT
EGF	TTGACAAGTGGCAGGAGGTC	TGGGCAGGAAACAAGTTCGT
TLR4	CGCTGCCACCAGTTACAGAT	CTTCAAGGGGTTGAAGCTCAG
IL-6	ACTTCACAAGTCGGAGGCTT	TGTGACTCCAGCTTATCTCTTGG
IL-10	TGGACTCCAGGACCTAGACA	CAAGTGTGGCCAGCCTTAGA
IL-1 β	TGCCACCTTTTGACAGTGATG	AAGGTCCACGGGAAAGACAC
TNF-α	AGGCACTCCCCCAAAAGATG	CCACTTGGTGGTTTGTGAGTG

Key Laboratory for Modification of Chemical Fibers and Polymer Materials, College of Materials Science and Engineering and Biomaterials and Tissue Engineering Lab, College of Chemistry, Chemical Engineering and Biotechnology, Donghua University [31]. The EEA was diluted with a 0.1 M NaOH solution to a concentration of 20 mg/l into a total volume of 100 ml. 10 ml of the supernatant overnight was then taken out, with 20 ml 5*PBS, 4 ml extract of MSCs and 66 ml water. The pH of the mixture was adjusted to 7.4 by filtered sterilization. In a sterile chamber, 0.1 g of hyaluronic acid was added, mixed, and set aside in 5ml of sterile syringe at 4°C for 24 h. Then it was put into a sterile bag and can be stored at 4°C for approximately 1 month.

Construction of animal models

A 2 cm diameter piece of skin was excised from 6-week-old nude mice, under anesthesia, from the back, with cotton thread suturing of the edge to prevent wound contraction. Application of the EEA-MSC gel on the wound was followed by coverage of the wound with gauze. The EEA-MSC gel was injected and applied to the wound once a day, using 1 ml gel per mouse at one time. The gauze was opened 72 h later, and the cotton thread was taken out so that the wound could grow naturally. The AF-MSC was injected onto the wound once a day until recovery.

RNA extraction, reverse transcription polymerase chain reaction (RT-PCR) and quantitative real time polymerase chain reaction (qPCR)

Total RNA extraction, RT-PCR and qPCR assays were performed from the different cell lines mentioned earlier. Briefly, after collection, the total RNA of the cells and tissues was purified using Trizol reagent (Invitrogen 15596-026 Carlsbad, CA, USA) following the manufacturer's protocol and was reverse-transcribed in a 20 µl oligo random primer system (Takara RR-064A Shiga, Japan).The resulting cDNA of the samples was amplified under the following conditions: 95°C for 10 s for denaturation, 95°C for 10 s and 60°C for 30 s for 40 cycles using the LightCycler 480 II (Roche Basel, Switzerland). Primer sequences are shown in Table 1.

Western blotting

The proteins were extracted using 200 µl M-PER Mammalian Protein Extraction Reagent (Pierce, IL, USA) from approximately 1×10^6 cells of 20 mg tissue. The resultant suspension was collected and subjected to centrifugation at 10,500 *x* g at 4°C for 15 min. Bicinchoninic acid (BCA) assay (Pierce) was carried out to quantify the concentrations of total protein in the samples. 20 µg of protein underwent subsequent electrophoresis through a 10% SDS-polyacrylamide gel. Separated proteins were transferred to a 0.2 mm polyvinylidene fluoride membrane (PVDF; Millipore, MA, USA) using a Bio-Rad semi-dry blotting instrument. After blocking with 5% Bovine serum albumin (BSA) in triethanolamine-buffered saline solution (TBS) containing 0.1% Tween-20 for 1 h at room temperature, the membranes were incubated with a primary antibody including VEGFA (rabbit anti-mouse, Abcam, Ab9485), FGF2 (rabbit anti-mouse, Abcam, Ab92337), EGF (rabbit anti-mouse, Abcam, Ab184265), GAPDH (rabbit anti-mouse, Abcam, Ab9485), IL6 (rabbit anti-mouse, Abcam, Ab259341), IL10 (rat anti-mouse, Abcam, Ab189392), TLR4 (rabbit anti-mouse, Abcam, Ab13556) and β-actin (rabbit anti-mouse, Abcam, Ab8227), as described previously [32]. After several washes, the membranes were incubated with a secondary antibody and visualized using the enhanced chemiluminescence (ECL) western blot system. Quantification of western blots was performed through densitometry using Mini-Protean 3 (System Bio-Rad,Hercules, CA, USA).

Hematoxylin & eosin staining

For histopathological analysis, the formaldehyde-fixed, paraffin-embedded tissues were affixed, and hematoxylin and eosin (H&E) staining was done as previously described [33]. Histopathological changes were observed with a light photomicroscope (Leica, Wetzlar, Germany).

Immunofluorescence

The sample cells were fixed using 4% paraformaldehyde for 10 min and further permeabilized with 0.4% Triton X-100. Primary antibodies were added into the cells for incubating overnight at 4°C. The secondary antibodies were added into tubes to incubate for 1 h, and then a DAPI solution was added to counterstain the cell nuclei for an additional 20 min at 37°C [34]. Finally, the sample cells were observed with a fluorescence microscope (Leica).

Image flow cytometry test

Cells were collected and analyzed via imaging flow cytometry for CD11b (Pe-Cy5) and CD45 (FITC) pan-staining (Miltenyi Biotec, Bergisch Gladbach, Germany). After incubation, 3–5 million cells were fixed and immunostained with the fluorescent antibodies. The sample cells were incubated for 1 h at room temperature in the dark. Subsequently, the incubated cells were washed using cold PBS and stained with 3 µm DAPI (Sigma-Aldrich Technologies, MA, USA). At least 15,000 cells of each sample were captured using the ImageStream mkII (Amnis, Inc., WA, USA) using a 40x objective. Three lasers, 405, 488 561 nm, and a charge-coupled device (CCD) camera were used to analyze DNA, CD45, CD11b and cell morphology, respectively. Images of cells were acquired at low speed.

ELISA test

Blood samples were extracted from the antecubital vein using aseptic precautions and collected in Vacutainer™ serum separator tubes containing spray-coated silica and clot activator and serum separator gel for serum separation. Post-centrifugation, serum was collected using a micropipette and stored at -80°C in Eppendorf tubes for further testing at a later date. Target proteins were estimated with sandwich ELISA kits supplied by Abcam™ (Abcam, Cambridge, UK). Protein levels in serum were estimated using a linear regression curve, which was plotted using the standards supplied by the manufacturer with the ELISA kit.

Statistical analysis

The experimental data were statistically analyzed by statistical analysis software (SPSS 19.0, IL, USA). The normal distribution of data was first identified by using the Kolmogorov–Smirnov test. Then, for quantitative variables with normal distribution, one-way analysis of variance (ANOVA) and the Student's t-test were performed; for quantitative variables without normal distribution, the non-parametric test (Kruskal–Wallis) was used. There was a statistically significant difference in data at $p < 0.05$.

Results

Extracts of Wharton's jelly-derived MSCs promote skin wound repair but are subject to interference

Under ethical approval, the authors harvested umbilical cord tissue from volunteers and prepared umbilical cord-derived MSCs, which were identified by marking positive CD73 and CD90, and negative CD45 and Human Leukocyte Antigen-DR isotype (HLA-DR), according to methods previously described (Supplementary Figire 1) [29,32]. The MSC extracts were obtained by lysis following the aforementioned method (MSC extraction and identification). The authors found that via comparisons of the extracts from cells such as HEK293T and 3T3, this extract is rich in a variety of cytokines, such as VEGFA, FGF2 and EGF (Figure 1A). After the addition of this extract into the culture systems of mouse embryo fibroblasts (3T3 cells) and human epidermal cells, a significant increase in cell proliferation could be detected, showing that MSCs can promote the proliferation of fibroblasts (Figure 1B). Subsequently, the authors utilized this extract from the MSCs for the treatment of mouse skin wounds. Results showed that the skin repair rate had been significantly enhanced. The time for coverage of the skin wound with granulation tissue decreased from 120 h to approximately 96 h (Figure 1C). In the tissue covered after 14 days, the newborn skin in the MSC group had folded skin with hyperpigmentation (Figure 1D), suggesting that the skin tissue was rich in new blood vessels. Aberrant scar tissue was presented after MSCs treatment.

The authors further noted that the inflammatory response could significantly interfere with the repair of the skin wound. Imiquimod, a Toll-like receptor (TLR)7 ligand, is used to treat various skin malignancies; it activates

10.2217/rme-2021-0076

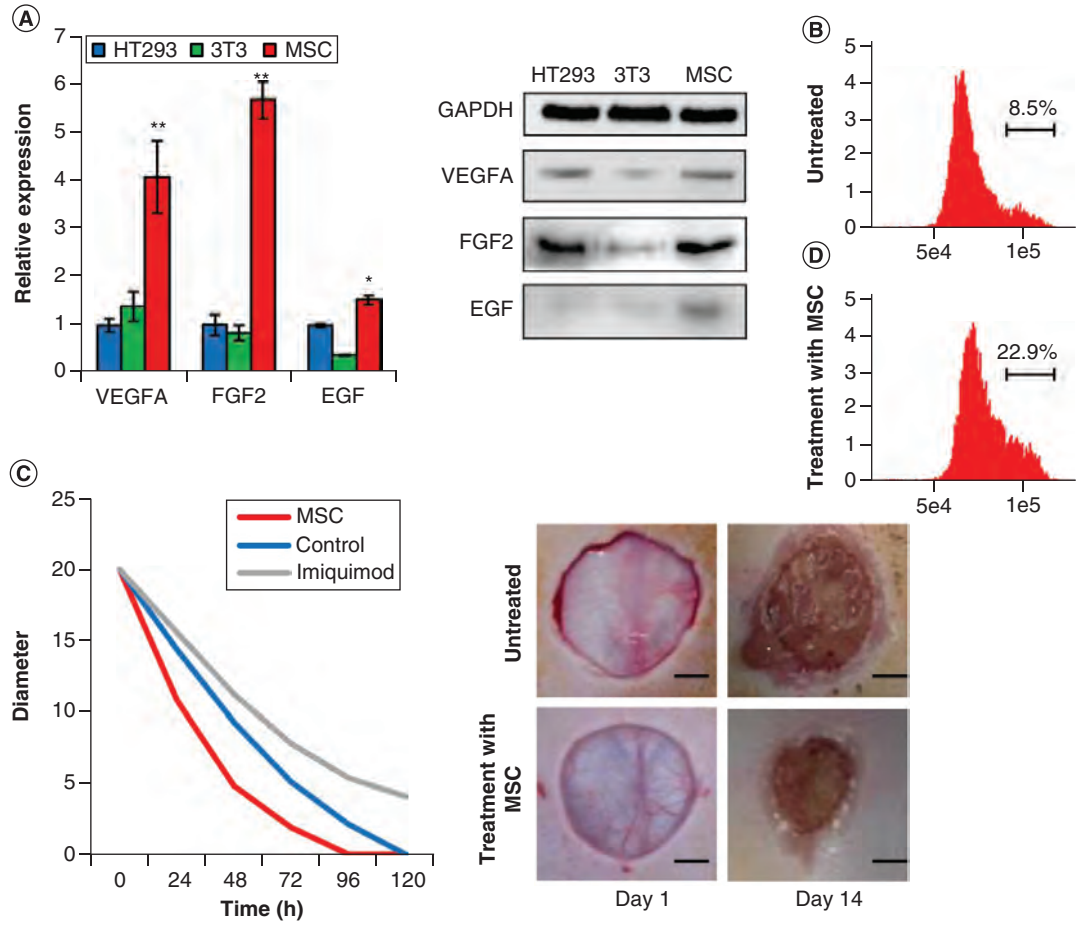


Figure 1. Mesenchymal stem cell (MSC) extract is conducive to skin wound recovery but subject to inflammation.
(A) The cytokine content of the protein supernatant of different cells (HEK293T, 3T3, MSC). Cell supernatants and cells were quantified using ELISA (left) and western blot (right), respectively. *$p < 0.05$; **$p < 0.01$. **(B)** MSC extract stimulated 3T3 cell proliferation, as assessed by flow cytometry **(C)** Treated Sprague Dawley rats with MSC extract, showing that the MSC extract can promote skin wound recovery (lower right panel). Scale bar = 1 cm. **(D)** Hematoxylin and eosin staining of fresh skin, showing that MSC extract can effectively promote skin regeneration, but this process was inhibited under imiquimod stimulation. Scale bar = 200 μm.
MSC: Mesenchymal stem cell.

both the innate and the acquired immune systems. Imiquimod induces the production and secretion of various pro-inflammatory molecules through stimulation of TLR7 on the surface of antigen-presenting cells, resulting in a delay of wound healing. When imiquimod was applied, wound recovery was affected, with a delay in the cover time by the granulation tissue (Figure 1C). The immunofluorescence results also showed significant epidermal tissue structure incompleteness, indicating the inhibition of the reagent on skin regeneration.

EEA inhibits inflammation & promotes skin wound healing

To deduce the cause of poor restoration by imiquimod, the authors analyzed granulation tissue using immunofluorescence assays. They revealed that an increased number of CD11b cells were present in abnormal granulation tissue at 16.7% versus normal tissue at 16.1% in the administration of MSCs, while a huge difference was observed in imiquimod treatment at 11.3% in normal tissue and 41.6% in abnormal recovery. The authors have speculated that poor trauma restoration could be associated with the aggregation of activated macrophages secreting large amounts of inflammatory cytokines (Figure 2A).

In previous studies, EEA was thought to have good anti-inflammatory effects. Experiments were performed in the macrophage cell line RAW264.7. These experiments confirmed that EEA inhibits the activation of the

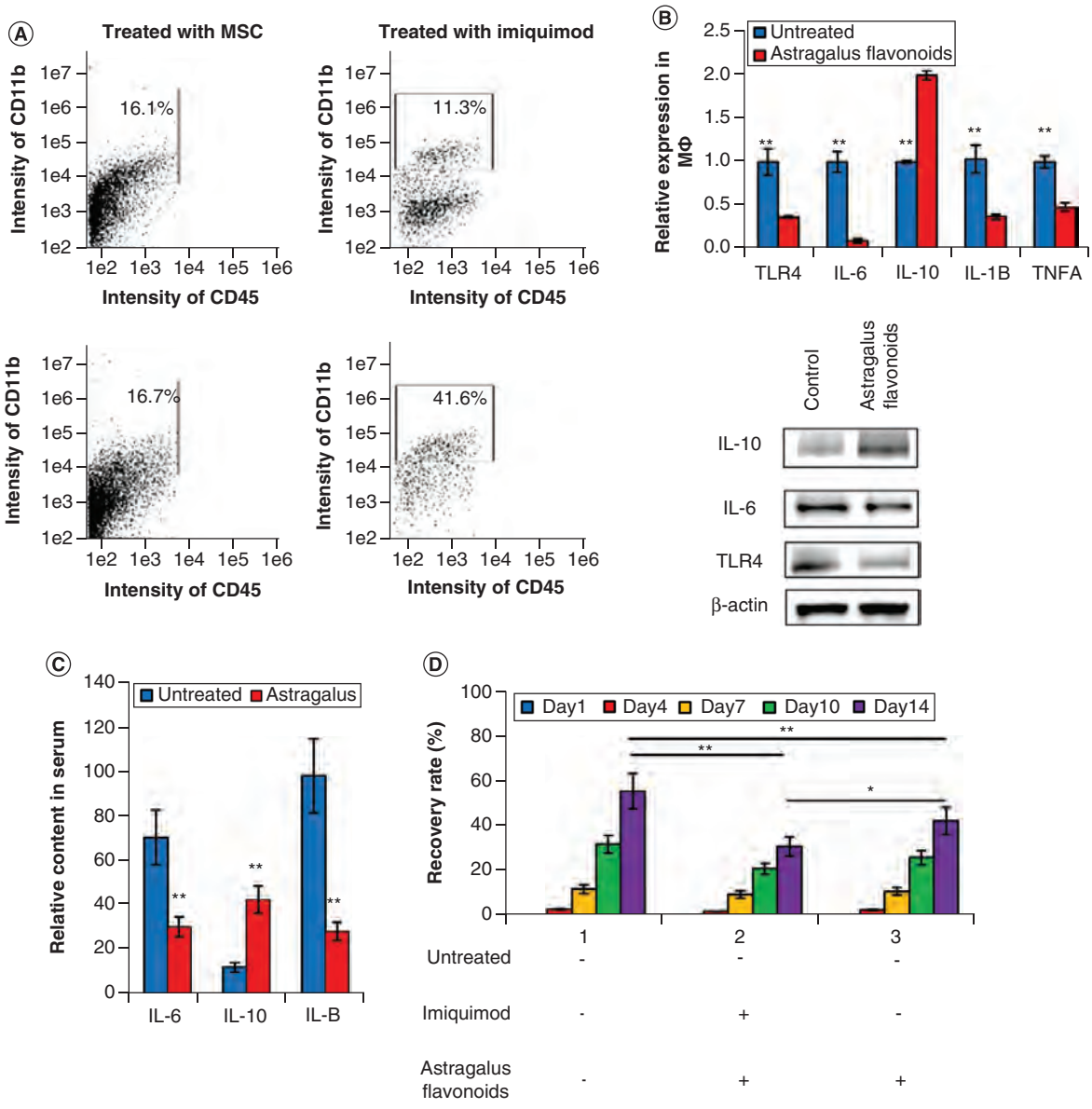


Figure 2. Ethanolic extract of astragalus can inhibit inflammation. (A) Flow cytometry analysis was performed on the cells of the wound site, and more CD11b+ macrophages could be found in tissue with low regeneration speed. **(B)** Stimulated macrophage cell line RAW264.7 with ethanolic extract of astragalus (EEA); downregulation of inflammatory factors, such as TNFA, IL-1β and IL-6, could be found. Upper panel: qPCR, lower panel: western blot. **(C)** Skin wound model with local inflammation using imiquimod and treated with EEA; inflammatory factors in peripheral blood serum were significantly reduced by ELISA. **(D)** Treatment of skin wound model with local inflammation using EEA, showing that the skin regeneration rate had been restored.
MSC: Mesenchymal stem cell; qPCR: quantitative Real-Time PCR.

ERK pathway in the macrophage cells stimulated by lipopolysaccharide (LPS), thereby reducing the expression of inflammatory factors such as TNF-α, IL-1β and IL-6 and increasing IL-10 (Figure 2B).

Animal experiments have confirmed that the treatment was effective. In the imiquimod-induced ulcer model of mice, when treated with EEA-MSC gel (containing MSC extract, EEA and the phosphate buffer system) in the wound surface, the aggregation rate of the macrophages was lower than that of the control group. In the peripheral blood, the contents of IL-1β and IL-6 were significantly decreased (Figure 2C). Although wound recovery had not returned to normal after using EEA, there had been some improvements (Figure 2D). This shows that EEA can reduce inflammation and thus could be helpful in wound recovery.

10.2217/rme-2021-0076

EEA promotes skin wound restoration

To test the effect of EEA in combination with MSC exosomes, MSC exosomes were mixed with EEA (EEA with 2 mg/l and 4% MSC extract) and applied as the treatment in a murine model. Results showed that this mixture helped in trauma restoration. The amount of time taken by the granulation tissue to reach total coverage was reduced to approximately 89 h. After 14 days of treatment, the mixture treatment group recovered faster than the imiquimod-induced inflammation group, and the healing time was much longer than that of the untreated group (Figure 3A).

The treated mice were culled at approximately 120 h, and the skin tissue was excised for further analysis. After staining the vascular cells with CD31 antibody, the mixture treatment group had an increased amount of vascular tissue, with an even higher microvessel density (MVD) than the treatment group that just had the MSC extract (Supplementary Figure 3). Although CD31+ cells were found in the skin tissue of the control group, the blood vessels had not formed a vascular network (Figure 3B). After treatment of the mice with the mixture, MVD increased in the granulation tissue, while IL-6 and IL-1β concentration decreased in peripheral blood (Figure 3C). These phenomena showed the anti-inflammatory effect of EEA. They also suggested a positive association between immunosuppression and angiogenescity. HE staining showed that the mixture treatment resulted in less scar tissue formation in the dermis layer, while the control group presented chaotic granulation tissue without differentiation on the wound site (Figure 3D). It is worth mentioning that there were fewer fibroblast cells in the granulation tissue of the mixture treatment group. This explained why the mixture treatment resulted in less scar tissue, as an excessive accumulation of fibroblasts is one of the major sources of scarring.

To illustrate this principle, the mixture was used to treat different skin-related cells, including HUVECs, Ha-cat and fibroblasts. When the concentration of EEA was 0.4% and above, fibroblasts were significantly inhibited, but this phenomenon was barely observed in HUVECs and epidermal cells (Figure 3E). This shows that EEA specifically inhibits fibroblast cells, thus reducing scar formation.

The slow-release gel can effectively enhance the role of MSC extract & EEA combination

Because skin wound repair occurs in an exposed environment, trauma treatments are susceptible to the influence of environmental factors [33]. MSC cytokines are not stable in a room-temperature environment. When MSC extract is applied to the skin in the form of the aqueous solution, its microenvironment rapidly changes with the evaporation of water, resulting in loss of activity, and when added to a liquid for use as a treatment, its effectiveness will be reduced, and the liquid-phase solvent will not work for a long time. Therefore, the authors considered taking advantage of a slow-release gel for treatment.

A chitosan-glucan gel was developed to solve this dilemma. This gel has high structural strength and can be stable for a long time *in vivo* and was thus used in this research to carry the MSC extracts and EEA mixture (Figure 4A). After a full analysis of the properties of the gel, it became apparent that it could play an important role in delivering sustained release of the mixture. In room-temperature environments (22°C), separating the supernatant of the gel and quantifying TGF-β1 showed 30% of the concentration after 48 h and 6.9% of the concentration after 144 h. Meanwhile, the control group showed 6.7% of the concentration after 24 h and almost can't be detected at 144 h. Stable release of the EEA could be detected until the complete disintegration of the gel (120 h) (Figure 4B). This means the gel could release TGF-β1 and EEA and extend MSC work time from 24 to 72 h. This offers the possibility of continuous and effective treatment with the MSC extract and EEA combination.

The gel was then applied to the skin wound of the mice, and it was observed that the speed of granulation had been further enhanced. Even more crucially, although MSC extracts have been reported to promote skin regeneration, the actual therapeutic effect is less than that of EEA-MSC gels.

Discussion

Wound healing is a complicated and multifaceted physiological process to maintain the integrity of skin after trauma; it depends on the various types of cells presenting overlapping phases, including an inflammation phase, a proliferation phase and a remodeling phase [34]. Neutrophils and macrophages are the first-line cells to clean debris and phagocytose bacteria and damage tissues to provide a proper environment for wound healing in the first phase [35]. Remodeling is the last phase, involving the balance of fibroblast apoptosis and extracellular matrix (ECM) degradation. Aberration in any of the three phases, including continuously localized inflammation, fibroblasts' exaggerated function and excess accumulation of ECM, may lead to excessive wound healing or a chronic wound [34,36,37].

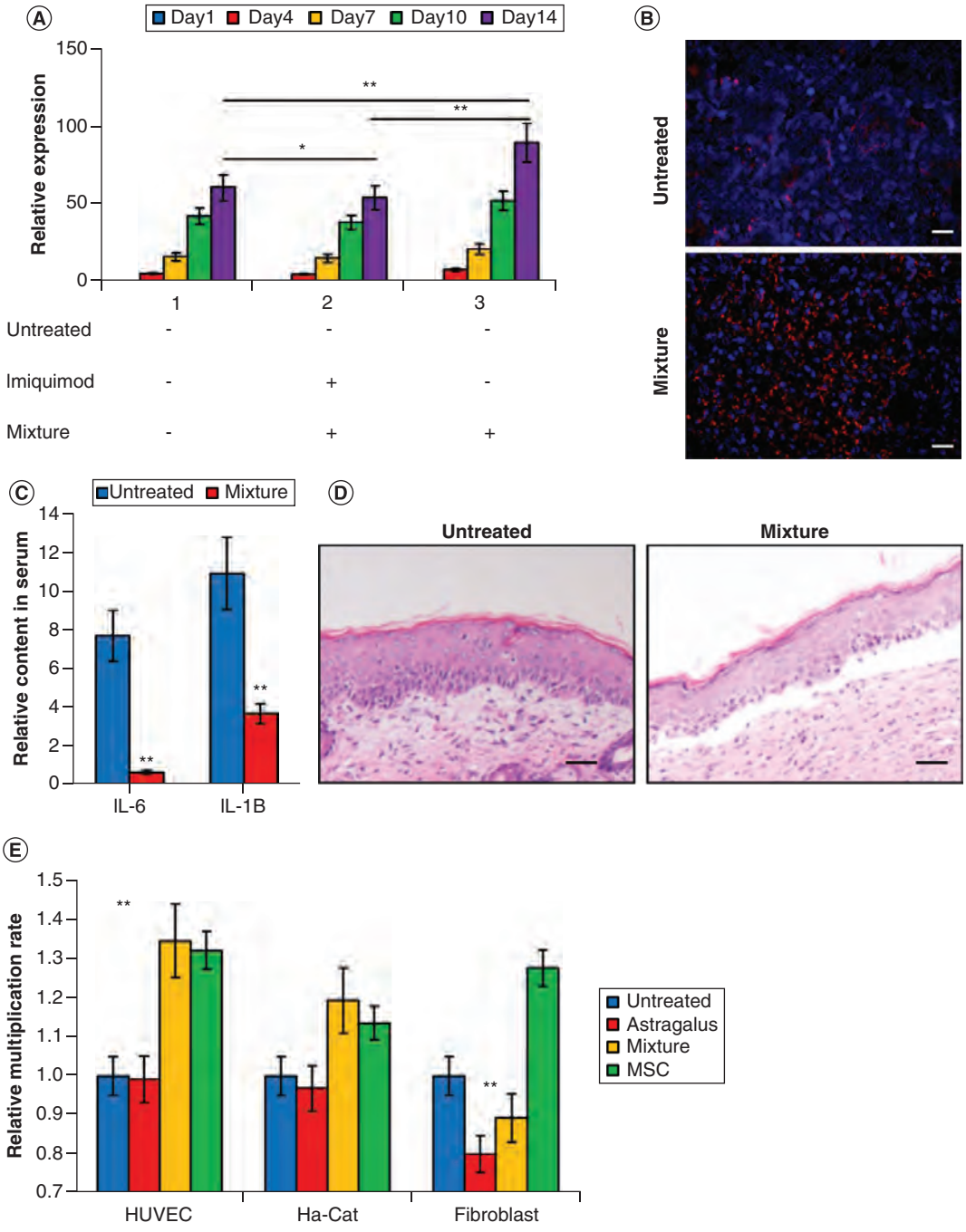


Figure 3. Mixing ethanolic extract of astragalus with mesenchymal stem cell extract can promote wound healing.
(A) Treated imiquimod model with a mixture of ethanolic extract of astragalus (EEA) and MSC extract, showing that the wound healing rate had improved. **$p < 0.01$. **(B)** Immunofluorescence detection for CD31, showing that after treatment with the mixture, vascular cells increased. Scale bar = 40 μm. **(C)** Treated imiquimod model with a mixture of EEA and MSC extract; little IL-6 and IL-1β, were detected in peripheral blood tested by ELISA. **$p < 0.01$. **(D)** Hematoxylin and eosin staining on the wound site; less scar tissue could be found in the treatment group (see arrow). Scale bar = 100 μm. **(E)** Cell proliferation of human umbilical vein endothelial cells, Ha-Cat and fibroblasts was determined by Cell Counting Kit 8. Fibroblasts were significantly inhibited in the treatment of 0.4% concentration of EEA; this phenomenon was negligible in human umbilical vein endothelial and Ha-Cat cells.
HUVAC: Human umbilical vein endothelial cell; MSC: Mesenchymal stem cell.

10.2217/rme-2021-0076

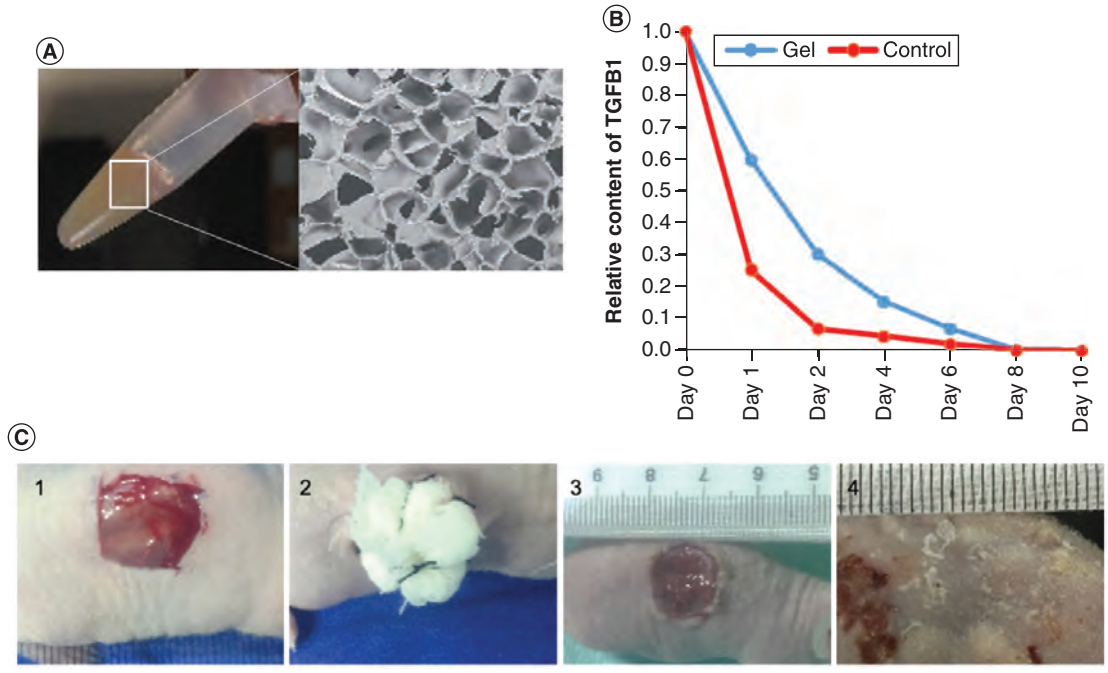


Figure 4. Dextran gel can promote the slow release of the active ingredient in the mixture. (A) Upper left: dextran gel with mesenchymal cell extract and ethanolic extract of Astragalus; lower left: dextran gel molecules under electron microscopy; right: dextran gel sprayed on the skin of a volunteer. **(B)** Changes in TGF-β1 content in the gel supernatant, showing the sustained-release effect of the gel. **(C)** Wound healing after a single application of dextran gel of ethanolic extract of astragalus-mesenchymal stem cell treatment.
1: Mouse skin wound opened with sprayed gel.
2: Wound fixed with cotton thread and covered with gauze.
3: Three days after treatment, the wound begins to heal.
4: Fourteen days after treatment, the wound is cured.

MSCs, a population of descendant progenitor cells that can self-renew and differentiate into multiple lineages, have gained much attention in skin repair and regeneration in recent years [3,5,12]. MSC-based therapy is currently a popular topic and efficacious in the medical profession, whereby it has been used for skin-regenerative and antifibrotic properties [37–43]. However, it is believed that MSC transplantation has some limitations: instability at room temperature, micro-infarctions, rejection reactions and other adverse events [33,44–50]. On the other hand, local inflammatory skin lesions can make wound healing worse, and excessive cell proliferation can lead to scarring and affect the recovery of wounds [4]. Emerging evidence has demonstrated that MSCs play a therapeutic role mainly through paracrine signaling, which affects the proliferation, migration and even survival of the neighboring cells [51–53]. The activity of effector cells such as inflammatory cells, endothelial cells, epidermal cells, keratinocytes and fibroblasts is strictly regulated by various growth factors and cytokines paracrined by MSCs,including IL, EGF, FGF, PDGF, TGF and VEGF. Exosomes are the critical bioactive entities that can regulate physiological and pathological processes of the recipient cells [54,55]. The present study's results have shown that MSCs secreted more VEGFA, EGF and FGF than HEK293 and 3T3. Among the growth factors generated by MSCs, VEGFA and FGF were elevated two- and three-times higher than EGF, respectively. VEGFA exerts its function in angiogenesis, and FGF stimulates the proliferation and differentiation of fibroblastic MSCs, promoting further cytokine secretion and participating in collagen deposition.

Astragalus, the dried root of *Astragalus mongholicus Bunge*, is one of the most popular traditional Chinese medicines and has been widely recognized as anti-inflammatory, antioxidative, anticancer, antidiabetic, cardioprotective, hepatoprotective and antiviral. More than 200 constituents can be isolated from the *Astragalus* genus and flavonoids, and EEA has been demonstrated to have potent activity in anti-inflammation and immunoregulation [56]. In the authors' findings, EEA could suppress the pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and TLR4 and promoted the anti-inflammatory factor IL-10. More importantly, the proliferation of fibroblasts

was curbed by EEA, which indicates that EEA might function in anti-inflammatory and antifibrotic processes and prevent excessive wound healing or extension of skin regeneration.

When the authors treated injured skin tissue with EEA and MSC extract together, they observed promoted CD31+ cells which was presented by microvessel density (MVD). Neovascularization or angiogenesis was the key step in the formulation of granulation tissue in the process of wound healing. Angiogenic pathways were characterized by elevation of the expression of VEGF, FGF and PDGF. The efficacy of the combination EEA and MSC extract treatment was also demonstrated by descending pro-inflammatory cytokine IL-6 and IL-1 β, which indicated a positive association between immunosuppression and angiogenescity. Imiquimod (IMQ) is a TLR7 agonist that is frequently used to treat superficial basal cell carcinoma (BCC), cutaneous squamous cell carcinoma and cutaneous malignant melanoma metastases. IMQ also exerts TLR7-independent effects to activate the inflammasome, trigger apoptosis and induce autophagic cell death in different cancer cells. Further, IMQ is an immune response modifier to cancerous skin lesions [57]. This study demonstrated that IMQ-induced immune dysfunction in mice could inhibit the effects by MSCs and cause excessive inflammation to delay the wound healing process.

This study offers two possible solutions. First, the use of cytokines from the umbilical cord-derived MSCs in combination with EEA, was found to have a positive anti-inflammatory function in a previous study, and thus offset the negative effects caused by local inflammation. Research shows that this additive can eliminate inflammation, improve the speed of healing and result in scar reduction after treatment. Further investigation found that the additive inhibits fibroblast proliferation, although this function is limited in epidermal cells and vascular cell function.

Second, gelatin was used as a stable carrier of MSC cytokines to allow their slow release. Through this, the effectiveness of cytokines was extended to 72 h. However, there are some limitations to this study. The reasons for the positive effect of EEA in this treatment are still unclear, including its role in inflammation receptors and specific inhibition of fibroblast proliferation. Their effect and safety in humans and animals need further work. Gelatin as a carrier also has great value for further study for its strength and potential as a treatment for other diseases, such as rheumatoid arthritis, and for its use within the field of stem cell therapy.

Conclusion & future perspective

In conclusion, we are optimizing EEA as an additive to enhance wound healing via the paracrine function of human WJ-derived MSCs, which can effectively speed up the process of wound repair and decrease aberrant scar tissue. Our study provides a novel cell-free approach based on the mixture of MSCs and EEA for wound healing.

Wound repair and regeneration are complicated processes involving the interaction among growth factors, inflammatory cells, fibroblasts, keratinocytes and epidermal cells. The application of cell-free therapy based on MSC-derived exosomes in the management of dermal wounds is currently of enormous interest. Our results implied that the inflammatory microenvironments substantially impact the process of wound healing. MSC-derived exosomes can effectively promote cutaneous wound healing in combination with EEA by suppressing the inflammatory response. This study pointed out that the anti-inflammatory strategy is crucial for the treatment with MSC-derived exosomes in wound healing.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/rme-2021-0076

Acknowledgments

The authors thank Ms Liu (Arachna Skin Biotechnology Center, Eston Cell Technology [Shanghai] Co. Ltd) for the assistance of the graphical abstract.

Financial & competing interests disclosure

This work was supported by the National Natural Science Foundation of China (Grant No. 81700069 and 81302580) and major project of Eighth Medical Center of Chinese PLA General Hospital (No. 2021ZD005). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Summary points

- The recovery from and regeneration of skin wounds are important to the maintenance of epidermal function.
- This study investigated the potential role of extracts from the combination of the herbal plant astragalus and mesenchymal stem cells (MSCs) in wound healing.
- A murine model of a skin wound was established and treated with a combination of extract of astragalus and exosomes of MSCs.
- The expression of growth factors such as VEGF, FGF2 and EGF were elevated after treatment with MSCs.
- Administration of ethanolic extract of astragalus decreased the expression of inflammatory factors such as TNF-α, IL-1β and IL-6 and increased the anti-inflammatory factor IL-10, as well as inhibiting the proliferation of fibroblasts.
- The combination treatment reduced the duration of skin granulation to approximately 89 h and hastened the recovery time, with a lighter scar.
- A slow-release gel with the mixture was developed to prolong the effect from 24 to 72 h by gradually releasing TGF-β1, which promotes wound repair.
- Ethanolic extract of astragalus could enhance the properties of cytokines secreted from MSCs by effectively increasing recovery speed and improving prognosis.
- The extract of astragalus promotes the efficacy of MSC-derived exosomes in skin repair and the combination could provide a potential solution for the treatment of skin wounds.

References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Beam JW, Buckley B, Holcomb WR, Ciocca M. National Athletic Trainers' Association position statement: management of acute skin trauma. *J. Athl. Train.* 51(12), 1053–1070 (2016).
2. Jaeger M, Wagman Y, Liran A *et al.* A literature review of burns in reconstructed breasts after mastectomy. *Wounds* 28(12), 422–428 (2016).
3. Takeo M, Lee W, Ito M. Wound healing and skin regeneration. *Cold Spring Harb. Perspect. Med.* 5(1), a023267 (2015).
4. Eming SA, Martin P, Tomic-Canic M. Wound repair and regeneration: mechanisms, signaling, and translation. *Sci. Transl. Med.* 6(265), 265sr266 (2014).

•• **This review provides current concepts in tissue regeneration and repair.**

5. Wood FM. Skin regeneration: the complexities of translation into clinical practise. *Int. J. Biochem. Cell Biol.* 56, 133–140 (2014).
6. Ferguson MW, O'kane S. Scar-free healing: from embryonic mechanisms to adult therapeutic intervention. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 359(1445), 839–850 (2004).
7. Anagnostou E. Translational medicine: mice and men show the way. *Nature* 491(7423), 196–197 (2012).
8. Ali N, Hosseini M, Vainio S, Taïeb A, Cario-André M, Rezvani HR. Skin equivalents: skin from reconstructions as models to study skin development and diseases. *Br. J. Dermatol.* 173(2), 391–403 (2015).
9. Hitchcock J, Savine L. Medical adhesive-related skin injuries associated with vascular access. *Br. J. Nurs.* 26(8), S4–S12 (2017).
10. Ud-Din S, Volk SW, Bayat A. Regenerative healing, scar-free healing and scar formation across the species: current concepts and future perspectives. *Exp. Dermatol.* 23(9), 615–619 (2014).
11. Babalola O, Mamalis A, Lev-Tov H, Jagdeo J. Optical coherence tomography (OCT) of collagen in normal skin and skin fibrosis. *Arch. Dermatol. Res.* 306(1), 1–9 (2014).
12. Caplan AI. MSCs: the sentinel and safe-guards of injury. *J. Cell. Physiol.* 231(7), 1413–1416 (2016).
13. Laschke MW, Mussawy H, Schuler S, Eglin D, Alini M, Menger MD. Promoting external inosculation of prevascularised tissue constructs by pre-cultivation in an angiogenic extracellular matrix. *Eur. Cell Mater.* 20, 356–366 (2010).
14. Kiang JG, Zhai M, Liao PJ, Ho C, Gorbunov NV, Elliott TB. Thrombopoietin receptor agonist mitigates hematopoietic radiation syndrome and improves survival after whole-body ionizing irradiation followed by wound trauma. *Mediators Inflamm.* 2017, 7582079 (2017).
15. Zhao R, Liang H, Clarke E, Jackson C, Xue M. Inflammation in chronic wounds. *Int. J. Mol. Sci.* 17(12), 2085 (2016).
16. Snyder RJ, Fife C, Moore Z. Components and quality measures of DIME (devitalized tissue, infection/inflammation, moisture balance, and edge preparation) in wound care. *Adv. Skin Wound Care* 29(5), 205–215 (2016).
17. Altman I, Lee IH, Burns MR, Rondelli D, Ennis WJ. Calcinosis cutis presenting in the context of long-term therapy for chronic myeloid leukemia: a case report and review of the literature. *Wounds* 27(2), 20–25 (2015).
18. Marini L, Odendaal D, Smirnyi S. Importance of scar prevention and treatment – an approach from wound care principles. *Dermatol. Surg.* 43(Suppl. 1), S85–S90 (2017).

19. Theunissen D, Seymour B, Forder M, Cox SG, Rode H. Measurements in wound healing with observations on the effects of topical agents on full thickness dermal incised wounds. *Burns* 42(3), 556–563 (2016).
20. Mitsukawa N, Higaki K, Ito N *et al.* Combination treatment of artificial dermis and basic fibroblast growth factor for skin defects: a histopathological examination. *Wounds* 28(5), 158–166 (2016).

• **FGF promotes angiogenesis and fibroblast proliferation in the artificial dermis.**

21. Bianchi A, Painter KJ, Sherratt JA. A mathematical model for lymphangiogenesis in normal and diabetic wounds. *J. Theor. Biol.* 383, 61–86 (2015).
22. Sorg H, Tilkorn DJ, Hager S, Hauser J, Mirastschijski U. Skin wound healing: an update on the current knowledge and concepts. *Eur. Surg. Res.* 58(1–2), 81–94 (2017).
23. Kwan PO, Tredget EE. Biological principles of scar and contracture. *Hand Clin.* 33(2), 277–292 (2017).
24. Kim JM, Lee JH, Lee GS *et al. Sophorae flos* extract inhibits RANKL-induced osteoclast differentiation by suppressing the NF-κB/NFATc1 pathway in mouse bone marrow cells. *BMC Complement. Altern. Med.* 17(1), 164 (2017).
25. Hu G, Wang J, Hong D *et al.* Effects of aqueous extracts of *Taraxacum officinale* on expression of tumor necrosis factor-alpha and intracellular adhesion molecule 1 in LPS-stimulated RMMVECs. *BMC Complement. Altern. Med.* 17(1), 38 (2017).
26. Mercurio DG, Wagemaker TA, Alves VM, Benevenuto CG, Gaspar LR, Maia Campos PM. In vivo photoprotective effects of cosmetic formulations containing UV filters, vitamins, *Ginkgo biloba* and red algae extracts. *J. Photochem. Photobiol. B* 153, 121–126 (2015).
27. Zhao B, Zhang X, Han W, Cheng J, Qin Y. Wound healing effect of an *Astragalus membranaceus* polysaccharide and its mechanism. *Mol. Med. Rep.* 15(6), 4077–4083 (2017).

•• **This study demonstrated that APS2-1 as a novel polysaccharide isolated and purified from *Astragalus membranaceus* can enhance wound healing through inhibiting the inflammation reaction.**

28. Fu J, Wang Z, Huang L *et al.* Review of the botanical characteristics, phytochemistry, and pharmacology of *Astragalus membranaceus* (huangqi). *Phytother. Res.* 28(9), 1275–1283 (2014).

•• **This review gave the authors the idea to choose ethanolic extract of astragalus to carry this whole study for the reason that it was reported to exert anti-inflammatory, antioxidant and immunomodulating functions.**

29. Huang P, Lin LM, Wu XY *et al.* Differentiation of human umbilical cord Wharton's jelly-derived mesenchymal stem cells into germ-like cells *in vitro*. *J. Cell Biochem.* 109(4), 747–754 (2010).
30. Tong Q, Zhu Y, Zhang D *et al.* MicroRNA quantitation during dendritic cell endocytosis using imaging flow cytometry: key factors and requirements. *Cell Physiol. Biochem.* 51(2), 793–811 (2018).

• **A part of the method applied in this study following the present authors' previous work that had been published in *Cellular Physiology and Biochemistry*.**

31. Pan JF, Yuan L, Guo CA *et al.* Fabrication of modified dextran-gelatin *in situ* forming hydrogel and application in cartilage tissue engineering. *J. Mater. Chem. B.* 2(47), 8346–8360 (2014).

•• **This research provided the technique of the dextran-gelatin to carry the product of the combination of extract of astragalus and exosomes of mesenchymal stem cells.**

32. Lv FJ, Tuan RS, Cheung KM, Leung VY. Concise review: the surface markers and identity of human mesenchymal stem cells. *Stem Cells* 32(6), 1408–1419 (2014).
33. Vozenin-Brotons MC, Mauviel A. [How to model the events in cutaneous fibrosis?]. *Med. Sci. (Paris)* 22(2), 172–177 (2006).
34. Lindley LE, Stojadinovic O, Pastar I, Tomic-Canic M. Biology and biomarkers for wound healing. *Plast. Reconstr. Surg.* 138(Suppl. 3), S18–S28 (2016).
35. Berman B, Maderal A, Raphael B. Keloids and hypertrophic scars: pathophysiology, classification, and treatment. *Dermatol. Surg.* 43(Suppl. 1), S3–S18 (2017).
36. Ashcroft KJ, Syed F, Bayat A. Site-specific keloid fibroblasts alter the behaviour of normal skin and normal scar fibroblasts through paracrine signalling. *PLoS One* 8(12), e75600 (2013).
37. Wang PH, Huang BS, Horng HC, Yeh CC, Chen YJ. Wound healing. *J. Chin. Med. Assoc.* 81(2), 94–101 (2018).
38. Jiao Y, Wang X, Zhang J, Qi Y, Gong H, Jiang D. Inhibiting function of human fetal dermal mesenchymal stem cells on bioactivities of keloid fibroblasts. *Stem Cell Res. Ther.* 8(1), 170 (2017).
39. Trohatou O, Roubelakis MG. Mesenchymal stem/stromal cells in regenerative medicine: past, present, and future. *Cell Reprogram.* 19(4), 217–224 (2017).
40. Gaur M, Dobke M, Lunyak VV. Mesenchymal stem cells from adipose tissue in clinical applications for dermatological indications and skin aging. *Int. J. Mol. Sci.* 18(1), 208 (2017).
41. Shin TH, Kim HS, Choi SW, Kang KS. Mesenchymal stem cell therapy for inflammatory skin diseases: clinical potential and mode of action. *Int. J. Mol. Sci.* 18(2), 244 (2017).
42. Shen SP, Liu WT, Lin Y *et al.* EphA2 is a biomarker of hMSCs derived from human placenta and umbilical cord. *Taiwan J. Obstet. Gynecol.* 54(6), 749–756 (2015).

43. Ho CH, Lan CW, Liao CY, Hung SC, Li HY, Sung YJ. Mesenchymal stem cells and their conditioned medium can enhance the repair of uterine defects in a rat model. *J. Chin. Med. Assoc.* 81(3), 268–276 (2018).
44. Wu P, Zhang B, Shi H, Qian H, Xu W. MSC-exosome: a novel cell-free therapy for cutaneous regeneration. *Cytotherapy* 20(3), 291–301 (2018).

•• **This study gave the authors the idea to use mesenchymal stem cell-exosome for the treatment of wound healing.**

45. Martín-Henao GA, Resano PM, Villegas JM *et al.* Adverse reactions during transfusion of thawed haematopoietic progenitor cells from apheresis are closely related to the number of granulocyte cells in the leukapheresis product. *Vox Sang.* 99(3), 267–273 (2010).
46. Shu Z, Heimfeld S, Gao D. Hematopoietic SCT with cryopreserved grafts: adverse reactions after transplantation and cryoprotectant removal before infusion. *Bone Marrow Transplant.* 49(4), 469–476 (2014).
47. Bachier C, Potter J, Potter G *et al.* High white blood cell concentration in the peripheral blood stem cell product can induce seizures during infusion of autologous peripheral blood stem cells. *Biol. Blood Marrow Transplant.* 18(7), 1055–1060 (2012).
48. Syme R, Bewick M, Stewart D, Porter K, Chadderton T, Glück S. The role of depletion of dimethyl sulfoxide before autografting: on hematologic recovery, side effects, and toxicity. *Biol. Blood Marrow Transplant.* 10(2), 135–141 (2004).
49. Calmels B, Lemarié C, Esterni B *et al.* Occurrence and severity of adverse events after autologous hematopoietic progenitor cell infusion are related to the amount of granulocytes in the apheresis product. *Transfusion* 47(7), 1268–1275 (2007).
50. Cordoba R, Arrieta R, Kerguelen A, Hernandez-Navarro F. The occurrence of adverse events during the infusion of autologous peripheral blood stem cells is related to the number of granulocytes in the leukapheresis product. *Bone Marrow Transplant.* 40(11), 1063–1067 (2007).
51. Caplan AI, Correa D. The MSC: an injury drugstore. *Cell Stem Cell* 9(1), 11–15 (2011).
52. Pittenger M. Sleuthing the source of regeneration by MSCs. *Cell Stem Cell* 5(1), 8–10 (2009).
53. Ortiz LA, Dutreil M, Fattman C *et al.* Interleukin 1 receptor antagonist mediates the antiinflammatory and antifibrotic effect of mesenchymal stem cells during lung injury. *Proc. Natl Acad. Sci. USA* 104(26), 11002–11007 (2007).
54. Zhang X, Yuan X, Shi H, Wu L, Qian H, Xu W. Exosomes in cancer: small particle, big player. *J. Hematol. Oncol.* 8, 83 (2015).
55. Zhang B, Wang M, Gong A *et al.* HucMSC-exosome mediated-Wnt4 signaling is required for cutaneous wound healing. *Stem Cells* 33(7), 2158–2168 (2015).
56. Hong F, Xiao W, Ragupathi G *et al.* The known immunologically active components of astragalus account for only a small proportion of the immunological adjuvant activity when combined with conjugate vaccines. *Planta Med.* 77(8), 817–824 (2011).
57. Cho JH, Lee HJ, Ko HJ *et al.* The TLR7 agonist imiquimod induces anti-cancer effects via autophagic cell death and enhances anti-tumoral and systemic immunity during radiotherapy for melanoma. *Oncotarget* 8(15), 24932–24948 (2017).